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CHANGES IN HAEMOLYMPH PROTEINS OF THE FALL ARMYWORM, *SPODOPTERA FRUGIPERDA* (J. E. SMITH), ASSOCIATED WITH PARASITISM BY THE BRACONID PARASITOID *COTESIA MARGINIVENTRIS* (CRESSON)

S. M. FERKOVICH, P. D. GREANY, and C. DILLARD
Insect Attractants, Behavior, and Basic Biology Research Laboratory, Agricultural Research Service, USDA, Gainesville, FL 32604, U.S.A.

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Abstract—Changes in haemolymph proteins of the fall armyworm, *Spodoptera frugiperda*, associated with parasitism by the parasitoid *Cotesia* (= *Apanteles*) *marginiventris* were monitored by sodium dodecyl sulphate polyacrylamide gel electrophoresis. As early as hour 4 after parasitization treatment, several electrophoretically slow-migrating, high-molecular-weight proteins were detected in the host's haemolymph. These proteins were detected earlier in haemolymph from parasitized larvae than in haemolymph from control larvae, and their concentrations were higher in heavily parasitized host larvae (≥ 3 eggs/host) than in lightly parasitized larvae (1 egg/host). Additionally, unique proteins that migrated electrophoretically with bovine serum albumin appeared in the haemolymph of parasitized larvae at hour 8 after parasitization treatment and were evident in haemolymph collected through to hour 64.

Key Word Index: Haemolymph proteins, fall armyworm, *Spodoptera frugiperda*, *Cotesia marginiventris*, SDS gel electrophoresis, parasitism

INTRODUCTION

Hymenopterous endoparasitoids are known to cause many physiological and biochemical changes in the host insect, an action Vinson and Iwantsch (1980) termed "host regulation." These changes include alterations—in growth, food utilization and developmental rate of the host—that are thought to benefit the developing parasitoid. Changes that have been reported in the host's haemolymph after parasitization include changes in specific gravity, freezing point depression, dry weight, hormone titres, plus differences in amino acid and protein concentrations and in the number of electrophoretically separable proteins (Baldwin and House, 1952; Corbet, 1968; Barras *et al.*, 1969; Brewer *et al.*, 1973; Vinson and Iwantsch, 1980; Dahlman and Green, Jr., 1981; Beckage, 1982; Beckage and Riddiford, 1982; Thompson, 1982).

In this report we describe changes in haemolymph proteins in the fall armyworm, *Spodoptera frugiperda*, in response to parasitism by *Cotesia marginiventris*. This braconid wasp is an endoparasitoid of several economically important species of noctuid pests of cotton and other crops (Boling and Pitre, 1970; Kunnalaca and Mueller, 1979). Despite its potential for reducing crop damage, *C. marginiventris* has not been used in biological control release programmes because of the great expense involved in its rearing by conventional means with insects as hosts. *In vitro* culture of the parasitoid, however, might make its use in insect control programmes economically feasible.

Among the many nutritional considerations of potential importance in the *in vitro* culture of parasitoids, a primary one is utilization of proteins. We have therefore undertaken studies on changes in haemolymph proteins of parasitized *Spodoptera frugiperda* larvae as background information for studies on *in vitro* culture of *C. marginiventris*.

MATERIALS AND METHODS

Host and Parasitoid Colony Maintenance

The host species, *S. frugiperda* (J. E. Smith), was mass reared on a pinto-bean-base diet at 25°C, 55% r.h. and 14L:10D photoregime (Leppla *et al.*, 1982; Leppla *et al.*, in press).

For routine colony maintenance, adult *C. marginiventris* (Cresson) were held in Plexiglas® (acrylic) cages (25 × 25 × 25 cm with a sock entrance) at 26°C, 60–70% r.h. and a 14L:10D photoregime. Undiluted honey was streaked on the inside of the cages to provide food. Water was provided in glass vials containing dental wicks. Host larvae (late-second or early third instars) were exposed to adult wasps (50 hosts: 25 wasps) for 1 h in plastic petri dishes (100 × 15 mm) under fluorescent lighting. The wasps were then removed to the rearing cages and the parasitized larvae were kept in petri dishes. There, they were provided food as needed, and newly formed cocoons were removed and transferred to adult rearing cages daily. To prevent cannibalism of parasitized larvae, unparasitized larvae, distinguished by their larger size, were removed.

Experimental procedures for host parasitization

For initial experiments involving haemolymph collection from parastizied *S. frugiperda*, third-stage host larvae (head capsule width 0.6–0.8 mm) weighing 1.5–4.0 mg were selected and exposed to adult *C. marginiventris* females; however, repeatable electro-

phoretic patterns of haemolymph were not obtained for either parasitized or control larvae. In subsequent experiments, host larvae of a precise age and even more precise weight range were selected. Thus, larvae that had moulted to the third instar within a 4-h period were maintained in petri dishes with diet for 16 h. At that time larvae weighing 3.0–4.0 mg were selected and placed together in a petri dish with adult wasps (2 hosts/wasp) under fluorescent lighting for 1 h. Larvae of the same age and weight as those exposed to parasitoids were selected for controls. The ratio of male to female wasps was not taken into account except in one experiment, the purpose of which was to compare haemolymph samples collected from lightly and heavily parasitized hosts (1 and ≥ 3 parasitoids/host). In that experiment, the adult *C. marginiventris* were sexed and only females were used. After exposure to the hosts, the wasps were returned to the Plexiglas cages, where they were maintained for 24 h before being used to parasitize additional larvae.

Collection and preparation of haemolymph

Haemolymph samples were collected on hour 1, hour 4 and hour 8 after larvae were given control treatment or treatment with the wasps. Thereafter, collection was made at 8-h intervals until hour 160. Haemolymph was released by clipping a proleg and was collected in a 5 μl glass capillary tube. The haemolymph was immediately diluted to 20% (v/v) with ice-cold homogenizing buffer (50 mM potassium phosphate, pH 8.0 containing 0.2 M sucrose, 1 mM phenylmethyl sulphonyl fluoride (PMSF) and 0.01% 1-phenyl-2-thiourea (PTU) in a 0.4 ml polyethylene centrifuge tube. Samples were centrifuged in a Beckman® Microfuge at 9380 *g* for 10 min to remove cellular material and then pooled by group: control and parasitized. Before samples from the parasitized group were pooled, however, each larvae source was dissected and examined for the presence of a parasitoid. In the experiment to compare haemolymph samples from lightly and heavily parasitized larvae, the larvae were dissected and then thoroughly examined for the presence of only one parasitoid egg or three or more eggs per host.

Protein assay

Protein concentration of samples was determined by the method of Hartree (1972) with bovine serum albumin as standard.

Wounding of larvae

To determine whether wounding *S. frugiperda* larvae would cause changes in haemolymph protein banding patterns similar to those caused by parasitization, the cuticle of 20 third-stage larvae (3.0–4.0 mg) was pierced in the mid-lateral area with a fine, sterlized dissecting pin. Haemolymph from these larvae was collected 8 h later and compared electrophoretically with that collected at hour 8 from parasitized and control larvae.

Electrophoresis

Haemolymph samples were separated in one dimension with 20% SDS-polyacrylamide slab gels according to Swanton *et al.* (1975). Initially, haemolymph was run on 5, 7.5, 12.5, 15 and 20% gels. At the 12.5% and lower gel concentrations, many of the low-molecular-weight polypeptides migrated with the bromophenol blue front. Therefore the 20% gel concentration, which gave the best overall resolution, was subsequently used to run all samples. Before electrophoresis, samples were heated for 3 min at 100°C in 1 mM dithiothreitol, 1% SDS and 5% glycerol. Generally, 100 μg of protein was applied per gel lane. The gels were stained with a 1% (w/v) solution of Coomassie Brilliant Blue® R-250 in 50% (w/v) trichloroacetic acid for 20 min then destained overnight with methanol–acetic acid–water (5:1:5 by vol). All chemicals were purchased from BioRad (Richmond, CA). The gels were sandwiched between two 1 mm thick glass plates to prevent desiccation and then were scanned densitometrically in a Shimadzu® Dual Wavelength TLC scanner (Model CS-910) at 555 nm. Because the background absorbance varied from gel to gel, the instrument was re-zeroed prior to each gel scan.

RESULTS

Electrophoretic comparison of haemolymph samples from parasitized larvae

Figure 1 shows the dynamic nature of the protein changes both in the control haemolymph and parasitized-host haemolymph (henceforth referred to as "parasitized haemolymph" for convenience). Perhaps the most evident difference between control and parasitized haemolymph was the difference in time of appearance of certain high-molecular-weight proteins. In the electrophoretograms these proteins appeared near the top of the gel (labelled a and b). These bands first appeared in the electrophoretogram of parasitized haemolymph sampled at hour 4, although they were the most intense (visually) in that of parasitized haemolymph sampled at hour 32. In contrast, these proteins did not appear in control haemolymph until hour 8 (very faint) and were quite evident at hour 24. Thus, their formation appears to have been hastened by the presence of the parasitoid. The proteins underwent further quantitative and qualitative changes in both control and parasitized haemolymph during the later stages of growth through the last sampling time, hour 160. Developmentally, most of the parasitized larvae did not moult into the fourth instar until hour 48, and fourth-stage larvae remained in that instar throughout the rest of the 160-h sampling period. Most of the control larvae moulted into the fourth instar at hour 40, fifth instar at hour 88, and sixth instar at hour 136; then, they remained in the sixth instar until the end of the 160-h sampling period.

Perhaps of greater significance was the appearance of two unique bands in the electrophoretograms of parasitized haemolymph. One was sharp and narrow, the other broader and diffuse (Fig. 1A, bands labelled c). These bands first appeared in the electrophoretogram of parasitized haemolymph sampled at hour 8 and were quite obvious in the electrophoretograms of samples collected through hour 64 (Fig. 1B). They were less distinct in the electrophoretograms of subsequently collected samples. Bands with mobilities similar to these were only

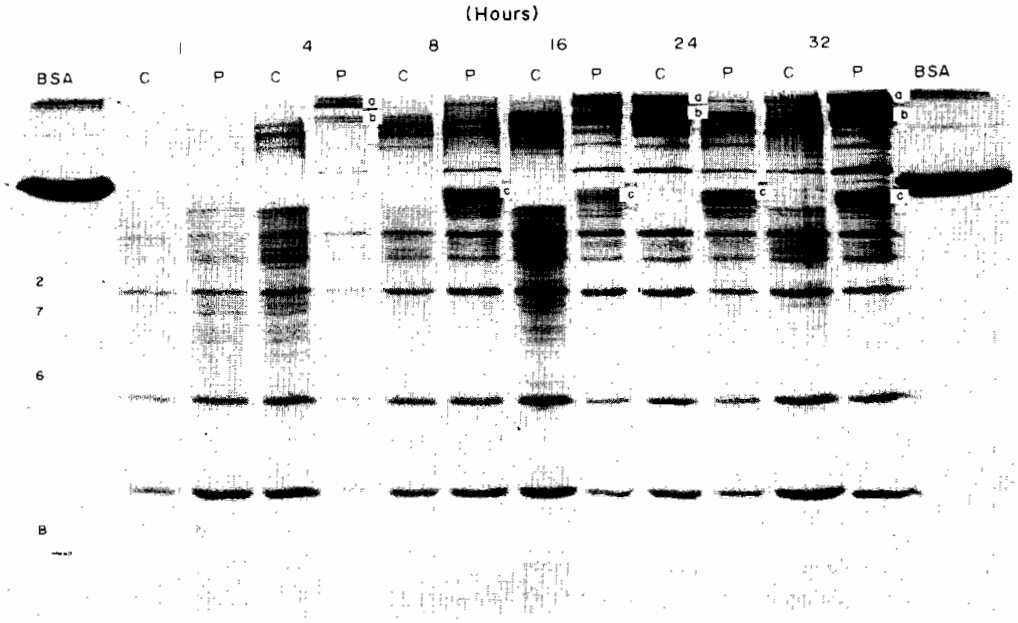
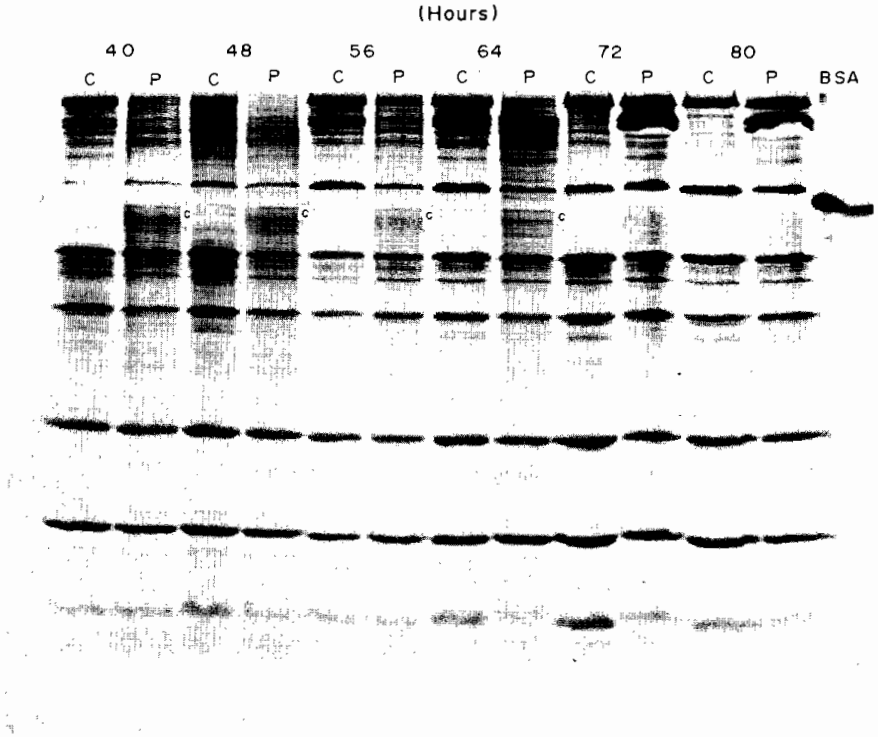

(Hours)
1
4
8
16
24
32
BSA
C
P
BSA
a
b
c
2
7
6
B

Fig. 1(A).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40
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56
64
72
80
C
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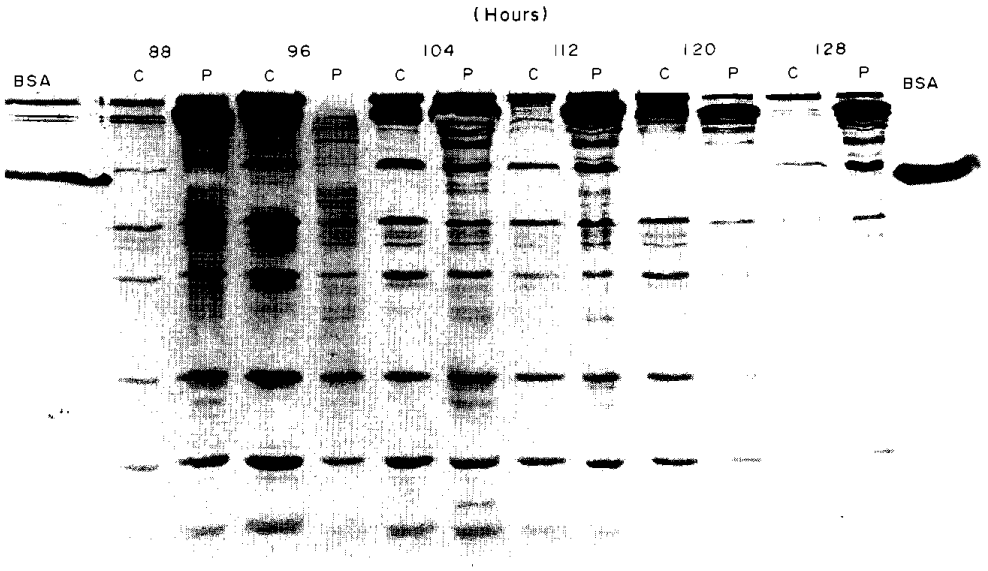
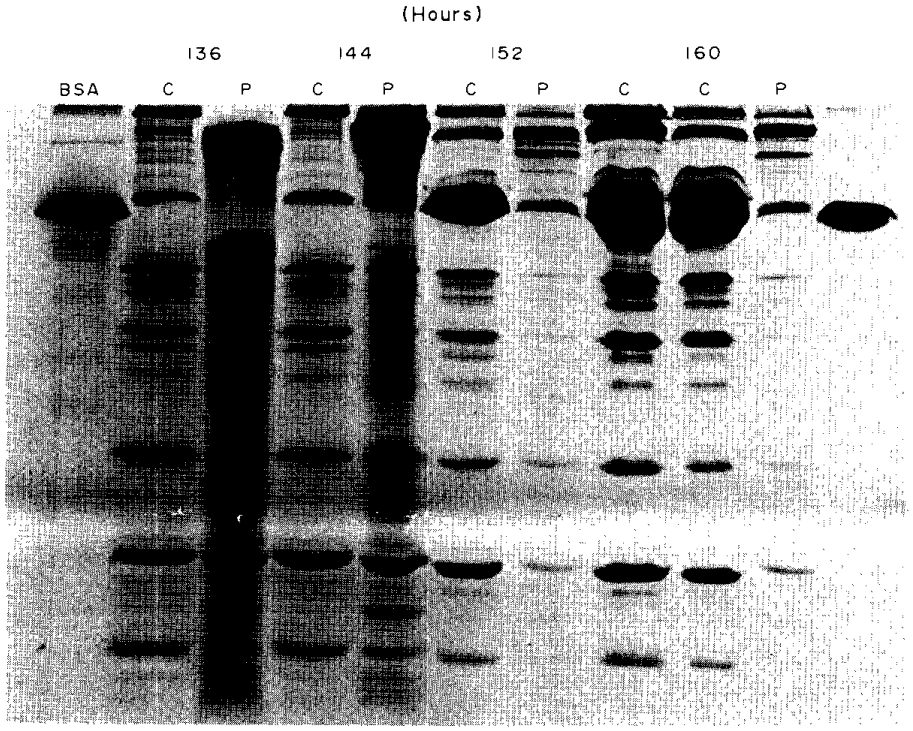
BSA
c

Fig. 1(B).



(C)



(D)

Fig. 1A, B, C, D. Electrophoretic patterns of haemolymph samples from control and parasitized larvae (20% SDS polyacrylamide gel). The larvae were stung in the early third instar by a parasitoid wasp, and haemolymph was collected 1 h through 160 h later. BSA, 20 μg bovine serum albumin was applied in extreme left and right lanes of each gel; C, control haemolymph (100 μg protein applied); P, haemolymph from parasitized larvae (100 μg protein). Letters along some of the gel lanes point out specific bands of interest (see text).

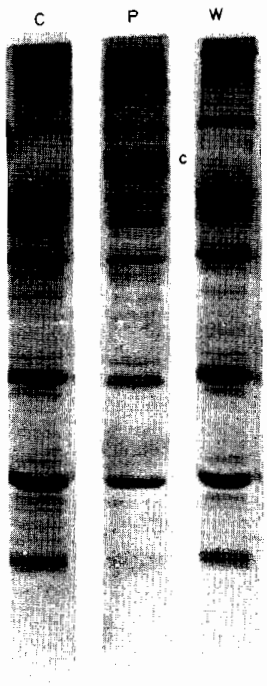


Fig. 2. Electrophoretic patterns (20% SDS polyacrylamide gel) of haemolymph (100 μg protein) from larvae 48 h after they had been subjected to control (C), wounding (W) and parasitization (P) treatments. Letter c indicates unique proteins in parasitized haemolymph.

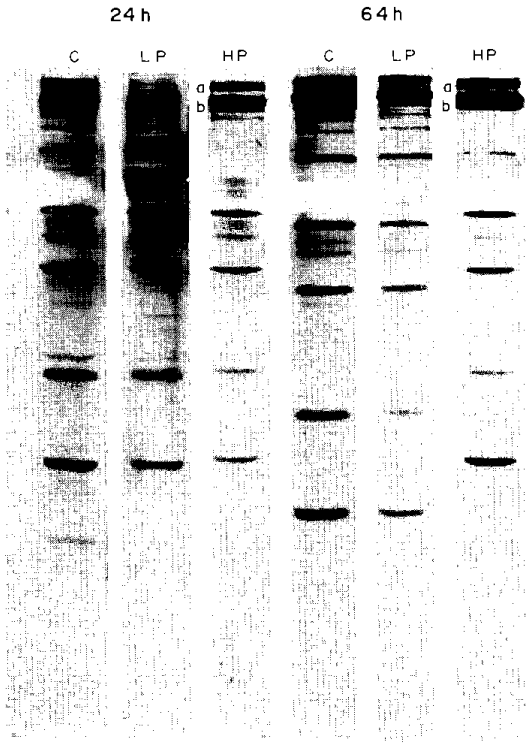


Fig. 3(A).

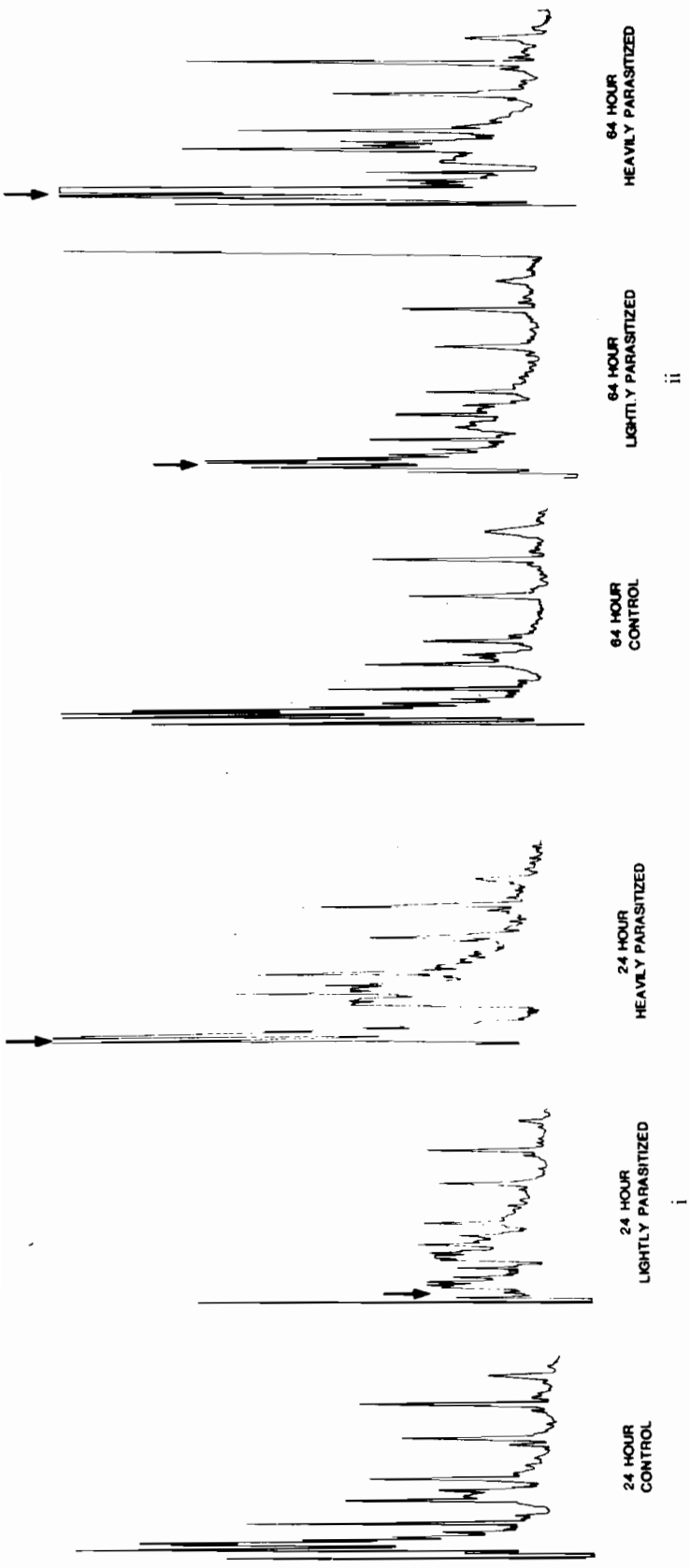


Fig. 3. A, Electrophoretic separations (20% SDS polyacrylamide gel) of haemolymph from larvae 24 and 64 h after they had been subjected to control (C), light parasitization (LP), and heavy parasitization (HP) treatments (100 μg protein applied). Letters a and b point out slow-migrating bands of interest. Lightly and heavily parasitized larvae contained 1 and ≥ 3 parasitoids/host, respectively. B, Densitometric scan of gels shown in A. Beginning of scan at gel surface corresponds to left side of the chart. Arrows refer to area of bands labelled a and b in A.

Table 1. Summary of reports on electrophoretic separation of proteins in haemolymph from parasitized hosts

Investigator	Host	Parasitoid	Electrophoretic separation medium	Parasitized host blood*		Host age	
				Number new bands	Number bands lost	Parasitization	Haemolymph collected
Fisher and Ganesalingam (1970)	*Ephestia kunehniella*	*Venturia canescens*	Cellulose acetate	2	0	First instar	Third instar
Vinson and Barras (1970)	*Heliothis virescens*	*Cardiochiles nigriceps*	7% PAG†	2	1	Second instar	Fourth instar
Barras *et al.* (1972)	*Heliothis zea*	*Microplitis croceipes*	7% PAG†	4	2	Second instar	3 days after parasitization
Smilowitz (1973)	*Trichoplusia ni*	*Hyposoter exiguae*	6% PAG†	1	2	Fourth and fifth instars	Fourth and fifth instars 2–6 days old
Brewer *et al.* (1973)	*Heliothis zea*	*Cardiochiles nigriceps*	7% PAG†	3		Fourth instar	Fourth instar
Smilowitz and Smith (1977)	*Pieris rapae*	*Apanteles glomeratus*	10% PAG†	0	6	Second instar	Third instar
Dahlman and Greene (1981)	*Manduca sexta*	*Apanteles congregatus*	4.5% PAG†	1	3	First instar	Third instar

*Compared with nonparasitized host haemolymph. †PAG indicates nondetergent polyacrylamide gels.

found in the electrophoretograms of control haemolymph at hour 48 in one experiment (Fig. 1B) and at hour 56 in a second experiment, but not at any of the other sampling times in either of the experiments.

Piercing the cuticle of control larvae to simulate oviposition by *C. marginiventris* females and then collecting haemolymph 8 h later resulted in the appearance of a few new bands in the electrophoretograms but none with exactly the same mobilities as those of bands c (Fig. 2).

The electrophoretograms of parasitized and control haemolymph also differed in the fast running bands, especially for haemolymph collected after hour 88 (Fig. 1C). In general, these bands were more numerous in the electrophoretograms of parasitized haemolymph than in that of control haemolymph; however, control larvae were already in the fifth instar whereas parasitized larvae were still in the fourth instar.

In some cases, the electrophoretic patterns were not always repeatable from one experiment to another. During the 1 h that the host larvae were exposed to *C. marginiventris*, we noticed that some larvae were attacked more than once. We therefore examined the effect of the level of parasitization on electrophoretic pattern. We found that electrophoretograms of haemolymph from lightly parasitized larvae (1 egg/host) generally contained bands of lower intensity, especially with regard to the slower migrating ones, than the electrophoretograms of haemolymph from heavily parasitized larvae (≥ 3 eggs/host) (Fig. 3A, B, 24-h bands a and b).

DISCUSSION

Several electrophoretic studies to identify parasitoid-induced changes in haemolymph proteins of host larvae are summarized in Table 1. In all studies cited, host haemolymph was collected 2 or more days from the time the larvae were parasitized and in some cases even from a later instar. In our study haemolymph was collected throughout the host-parasitoid association from as early as hour 1 up to as late as hour 168 after treatment of the host larvae with *C. marginiventris*.

As early as hour 4 after treatment, differences were observed between control and parasitized haemolymph, as indicated by their electrophoretograms. The primary difference was the earlier appearance of the high-molecular-weight proteins in areas a and b in the parasitized haemolymph. In addition, the concentration of these proteins also was higher in heavily parasitized larvae than in lightly parasitized larvae. The mechanism and significance of these changes in host haemolymph proteins in the parasitoid-host intraction remains to be determined.

The presence of the two new bands that consistently appeared in the electrophoretograms of parasitized haemolymph sampled at hour 8 prompt conjecture. These bands never appeared in the electrophoretograms of haemolymph from wounded, nonparasitized larvae. This suggested that in parasitized larvae the proteins were not released from the host's tissues due to the puncture wound made by the female wasp during oviposition. That bands with mobilities similar to these two were observed in the electrophoretograms of control haemolymph at two later sampling times (hours 48 and 56 in two separate experiments), suggested that the proteins were not unique to parasitized larvae. Further information is needed, however, to determine for certain whether these control proteins are similar to those found in parasitized haemolymph. Since their presence was first evident only 8 h after treatment, they might have been (1) released slowly from the egg (2) released from storage sites within the host tissues, or (3) synthesized *de novo* by the host. During oviposition, many braconid parasitoids, including *Apanteles* spp., also inject into the host larvae symbiotic baculovirus particles that invade the cell nuclei in various tissues (Stoltz and Vinson, 1979). This action raises the question at to whether the unique proteins are produced in response to baculoviruses. There is recent evidence that injection of calyx fluid containing baculoviruses from *Apanteles congregatus* (Say) females into larval *Manduca sexta* (L.) results in the appearance of a new polypeptide in the haemolymph of the larvae (D. Stoltz, personal communication). Whether similar actions involving the *C. marginiventris–S. frugiperda* association take place and, if they do, whether the proteins formed have a physiological effect on the association remain to be determined.

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